

Growth of *Salmonella enterica* Serovar Enteritidis in Albumen and Yolk Contents of Eggs Inoculated with This Organism onto the Vitelline Membrane

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ABSTRACT

By using an in vitro model simulating the potential opportunities for *Salmonella enterica* serovar Enteritidis (SE) to proliferate within eggs contaminated with this organism following oviposition, we investigated growth of SE in eggs. Seventy to 140 CFU of one of three SE strains originating either from egg contents, chicken meat, or a human infection were experimentally inoculated onto the vitelline membrane of eggs collected from specific-pathogen-free flocks of chickens and incubated at 25°C. SE organisms were detected in 6 of 71 yolk contents of the eggs inoculated with any of the test strains attaining levels ranging from 2.0×10^2 to 4.2×10^8 CFU/ml by day 6. The organisms were also detected in the albumen from 38 of 55 eggs tested, growing to levels ranging from 1.0×10^2 to 4.3×10^8 CFU/ml by day 6 after inoculation. An additional three yolk contents and 15 albumen samples were culture positive for SE following enrichment. There was no correlation between the number of the organisms in the yolk contents and that in the albumen from each of the eggs. When 73 to 91 CFU of the egg strain were inoculated into samples of separated albumen obtained from eggs that were stored at 4°C for 1 to 4 weeks or at 25°C for 1 week, slight growth (3.0×10^2 to 7.4×10^3 CFU/ml) was found in only 3 of the 60 albumen samples by day 6 after inoculation, but the organisms were recovered from 52 samples following enrichment. The results suggest that the environment on or near the vitelline membrane can be conducive to SE proliferation over time.

A large proportion of *Salmonella enterica* serovar Enteritidis (SE) infections in humans is the result of consumption of eggs contaminated with this organism. While most naturally contaminated eggs contain less than 10 cells of SE (12), problems can arise following the proliferation of SE in eggs postoviposition (7, 9, 19). When eggs were inoculated with SE under the shell membrane, the number of the organisms in the yolks of inoculated eggs increased by more than 8 log units in unrefrigerated eggs stored for 16 days, while growth of the microorganism was negligible in eggs refrigerated for the same period (10). Hammack et al. explained that migration of bacteria to the yolk creates an opportunity for growth of SE in yolk. Clay and Board (3) suggested that the rate and extent of infection were partly influenced by the site of contamination relative to yolk movement. Gast and Holt (7) confirmed that substantial SE multiplication often occurred when the bacteria had access to yolk nutrients. They also demonstrated (8) that SE inoculated onto the vitelline membrane surface of egg yolks was able to penetrate into and multiply within the yolk contents, although the number of SE in albumen was not evaluated. Results obtained by Fleischman and coworkers (5), however, indicated that a yolk membrane was impermeable to SE but permeable to yolk nutrients. Chicken egg vitelline membrane was found to be freely permeable to glucose both from albumen to yolk and from yolk to al-

bumen (6). Moreover, the membrane structure deteriorated during storage of the egg with the concomitant formation of degraded components of the membrane protein (16). Based on the evidence, SE inoculated close to the vitelline membrane may proliferate within the albumen in the absence of penetration of the vitelline membrane by the organisms. It is also unknown whether proliferation of SE in albumen alters the vitelline membrane dynamic to allow the penetration into the yolk. To clarify these points, the numbers of SE in both yolk contents and albumen were enumerated in eggs inoculated with the organism onto the vitelline membrane surface.

MATERIALS AND METHODS

Bacterial strains. SE KS2201 of phage type 1 was obtained from a patient during a food poisoning outbreak in 1995 (18). SE 333-12 of phage type 6a was recovered from egg contents in 1995 (20). SE 1116 of phage type 1 was isolated from retail chicken meat in 1999 in Japan. Each of the strains was grown in static culture to the stationary phase in tryptic soy broth (Becton Dickinson, Sparks, Md.) at 37°C. The culture was diluted in saline to a final concentration of approximately 1,000 CFU/ml, which was confirmed by spread plating 100 µl of the dilution onto brilliant green agar (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 0.01 mg/liter novobiocin (Sigma-Aldrich Corp., St. Louis, Mo.). Actual numbers of SE inoculated are described below.

Preparation of samples. Freshly collected eggs from specific-pathogen-free flocks of single comb white leghorn chickens

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at the Southeast Poultry Research Laboratory, U.S. Department of Agriculture were held at 4°C overnight before use. The eggs were aseptically broken, and the intact yolk sacs and albumen were separated and transferred to sterile plastic beakers. Each of the yolk sacs was inoculated with a 0.1-ml dose containing 70 to 140 CFU of SE on the exterior surface of its vitelline membrane and held at room temperature for 5 min, after which the albumen from a single egg was poured gently into the beaker to surround the yolk. Twenty-four samples inoculated with each strain were prepared and were incubated at 25°C.

Enumeration of *Salmonella* in samples. After 1, 3, and 6 days of inoculation, eight samples inoculated with each strain were sampled for the presence and number of salmonellae. Yolk contents were drawn as previously described (8). Briefly, each yolk sac and contents were transferred to a sterile petri dish, and a small area of the vitelline membrane was seared with the convex surface of a flame-heated steel spatula to destroy any bacteria present in that region. A 16-gauge syringe was inserted through the seared area to remove 3 ml of interior yolk contents (free of membrane). On day 6, an egg sample inoculated with the strain 1116 showed damage to the vitelline membrane and was discarded. Each sample was mixed with 3 ml of 2× tryptic soy broth. To determine the number of salmonellae in each yolk, the mixture was diluted fivefold in saline and then serially diluted 10-fold in saline, and 100-μl aliquots of each dilution were spread onto plates of brilliant green agar supplemented with 0.01 mg/liter novobiocin. The number of salmonellae in each albumen sample was determined by making serial dilutions in saline and spread plating 100-μl aliquots of each dilution onto plates of brilliant green agar supplemented with 0.01 mg/liter novobiocin. Nine milliliters of albumen from each sample was also removed and mixed with 1 ml of 10× tryptic soy broth and incubated at 37°C for 24 h as an enrichment step.

Growth of SE in separated albumen. Freshly collected eggs from specific-pathogen-free flocks described above were stored at 4°C for 1, 2, and 4 weeks or at 25°C for 1 week. Separated albumen was prepared by breaking surface-sterilized eggs into plastic beakers and removing the yolk. Each of the albumens was inoculated with a 0.1-ml dose containing 73 to 91 CFU of SE 333-12. After 1, 3, and 6 days of inoculation, five samples inoculated with the bacterium were tested for the presence and numbers of SE as described above.

Statistical analysis. Significant ($P < 0.05$) differences were determined among the numbers of SE found in albumen samples from different numbers of days after inoculation by application of the Mann-Whitney U test (21).

RESULTS AND DISCUSSION

Results for isolation and enumeration of salmonellae from the yolk contents and albumen of eggs inoculated with SE on the surface of vitelline membrane are shown in Figure 1. No SE organisms were detected in yolk samples from any of the samples tested at day 1 after inoculation. By day 3, SE organisms (1.4×10^3 CFU/ml) were detected in one of the samples inoculated with KS2201 and, following enrichment, from one sample inoculated with 1116. The enrichment method gave the positive result on day 3 for one sample inoculated with 1116. The positive result under the enrichment method was represented as $<1.0 \times 10^2$ CFU/ml because the detection threshold of the enumeration method was 100 CFU/ml. On day 6, SE organisms were

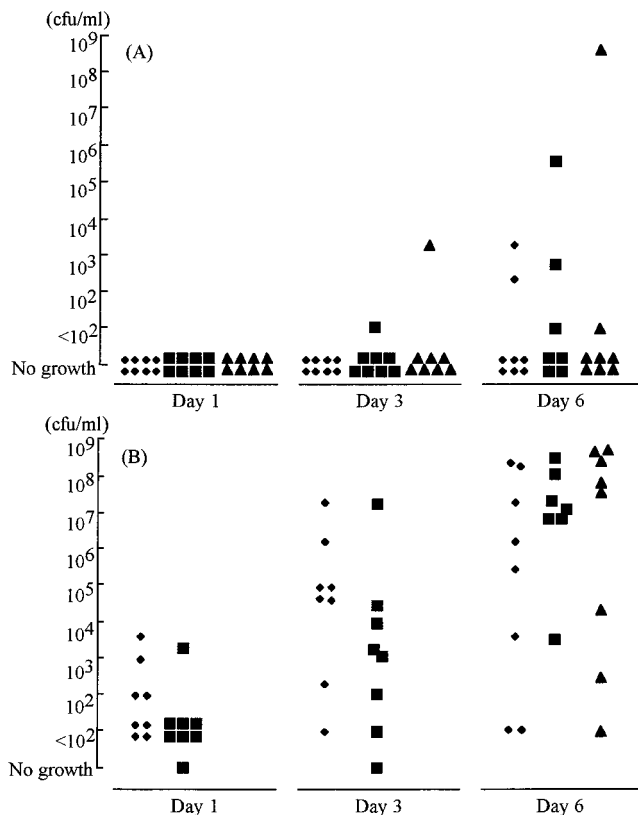


FIGURE 1. Isolation and enumeration of salmonellae from the yolk contents (A) and the albumen (B) of eggs inoculated with *Salmonella enterica* serovar *Enteritidis* 333-12 (diamonds), 1116 (squares), and KS2201 (triangles) on the surface of vitelline membrane. Isolation was not carried out from the albumen of eggs inoculated with KS2201 days 3 and 6 after inoculation.

isolated from each of two contents samples inoculated with strain 333-12 and KS2201, and from three samples inoculated with strain 1116. The numbers of SE in the contents samples ranged from 2.0×10^2 to 4.2×10^8 CFU/ml. A sample inoculated with strain 1116 and one with KS2201 were culture positive for SE following enrichment. Overall rate of penetration of SE through vitelline membrane in this experiment (9 of 71) was relatively low compared with the results from the previous report (8). The frequency of the penetration may differ as a result of the bacterial strains and eggs. Effects of antibodies against SE can be excluded because the eggs used in this study were obtained from the laboratory's specific-pathogen-free flocks and previous studies have failed to detect SE antibodies in eggs from unchallenged birds (11). Differences in the frequency due to strain origins were not evaluated in the present study because of the lack of sample size.

Relation between the number of salmonellae in the albumen and that in the yolk contents found in each of the same egg samples is shown in Figure 2. Eggs with high numbers of salmonellae in the albumen did not always show high numbers of the organism in the yolk contents. Braun and Fehllhaber (2) reported that the migration rate of SE from the albumen into the egg yolk in eggs with artificial contamination of the albumen with different doses of the organisms was positively correlated with the level of

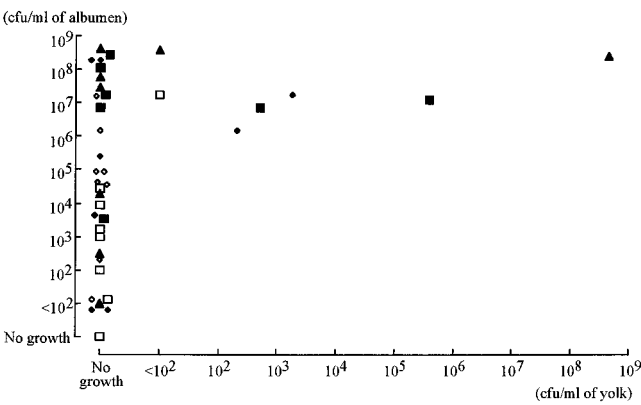


FIGURE 2. Relation between the number of SE in the yolk and that in the albumen from each egg obtained on days 3 (open symbols) and 6 (closed symbols) after inoculation. Eggs were inoculated with *Salmonella enterica* serovar *Enteritidis* 333-12 (diamonds) and 1116 (squares).

contamination. Nevertheless, the number of SE organisms observed in the contents of one yolk on day 6 was quite high (4.2×10^8 CFU/ml), indicating that the penetration might occur immediately after the inoculation in this egg.

SE organisms were isolated from 53 of the 55 albumen samples during the period of the experiment (Fig. 1B). Of the 53 samples, 15 were culture positive following enrichment. The numbers of SE in the remaining 38 samples ranged from 1.0×10^2 to 4.3×10^8 CFU/ml. The numbers of SE in the albumen samples inoculated with 333-12 on days 3 and 6 were significantly ($P < 0.05$) higher than that observed on day 1. The number in the albumen samples inoculated with 1116 on day 6 was higher than that on day 3 but not significant ($0.05 < P < 0.1$). These observations indicated that SE inoculated on the surface of vitelline membrane survived and proliferated in albumen.

Proliferation of SE in albumen can potentially result from the use of nutrient compounds emerging from yolk through the vitelline membrane (6, 16). To test this hypothesis, SE was inoculated into separated albumen from eggs stored for 1 to 4 weeks. Although survival of SE in 55 of the 60 separated albumen was observed, slight growth was found in only 3 of the 55 samples at concentrations ranging from 3.0×10^2 to 7.4×10^3 CFU/ml (Fig. 3). In the remaining 52 samples, SE was detected following en-

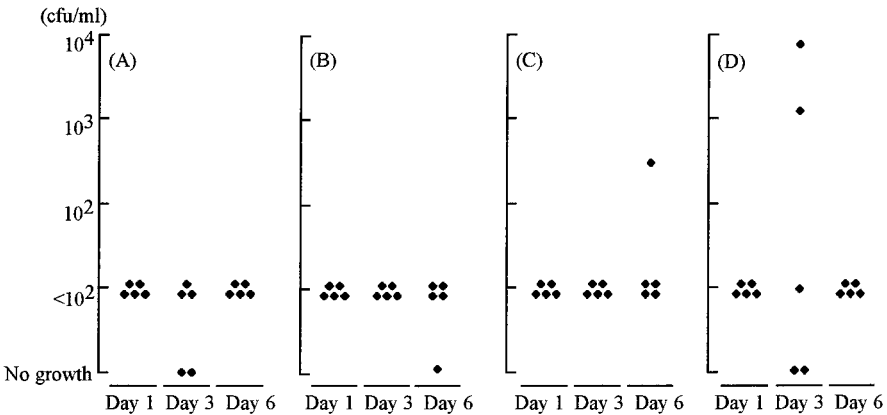
richment. The results suggest that the amount of nutrient elements in yolk that passed through the vitelline membrane to albumen during the storage was not enough to support the proliferation of SE. Cogan et al. (4) reported that growth of SE in separated albumen obtained from fresh eggs was unchanged when the bacteria was suspended in 0.2 ml of either buffered peptone water, maximal recovery diluent, or phosphate buffered water and inoculated.

The present study demonstrates that SE inoculated onto vitelline membrane could proliferate in albumen surrounding the yolk without penetrating the membrane into the egg yolk. It also suggests that only organisms on the surface of the vitelline membrane or those located nearby the membrane could proliferate using the nutrient elements passed from the yolk because growth of SE was rarely found in separated albumen obtained from stored eggs as described above. Unknown factors in the yolks might protect the organisms from various components in the albumen that provided antimicrobial barriers (1, 14, 15). Alternatively, genes essential for bacterial survival in egg albumen, such as *yafD*, which was identified recently (17), might be expressed only when the organisms were adjacent to the vitelline membrane. Since considerable prior investigations found that the albumen is more frequently contaminated with SE than yolk in eggs from naturally or experimentally infected hens (8, 13), mechanisms of survival and growth of SE in egg albumen need to be elucidated.

REFERENCES

1. Baron, F., M. Gautier, and G. Brule. 1997. Factors involved in the inhibition of growth of *Salmonella enteritidis* in liquid egg white. *J. Food Prot.* 60:1318–1323.
2. Braun, P., and K. Fehlhaber. 1995. Migration of *Salmonella enteritidis* from the albumen into the egg yolk. *Int. J. Food Microbiol.* 25: 95–99.
3. Clay, C. E., and R. G. Board. 1991. Growth of *Salmonella enteritidis* in artificially contaminated hens' shell eggs. *Epidemiol. Infect.* 106: 271–281.
4. Cogan, T. A., G. Domingue, H. M. Lappin-Scott, C. E. Benson, M. J. Woodward, and T. J. Humphrey. 2001. Growth of *Salmonella enteritidis* in artificially contaminated eggs: the effects of inoculum size and suspending media. *Int. J. Food Microbiol.* 70:131–141.
5. Fleischman, G. J., C. L. Napier, D. Stewart, and S. A. Palumbo. 2003. Effect of temperature on the growth response of *Salmonella enteritidis* inoculated onto the vitelline membranes of fresh eggs. *J. Food Prot.* 66:1368–1373.
6. Garcia, F. J., A. Pons, M. Alemany, and A. Palou. 1983. Permeability

FIGURE 3. Isolation and enumeration of salmonellae from separated albumen of eggs, which were stored at 25°C for 1 week (A), at 4°C for 1 week (B), at 4°C for 2 weeks (C), and at 4°C for 4 weeks (D) and were inoculated with *Salmonella enterica* serovar *Enteritidis* 333-12.



- of chicken egg vitelline membrane to glucose, carbohydrate gradients between albumen and yolk. *Comp. Biochem. Physiol. B* 75:137–140.
7. Gast, R. K., and P. S. Holt. 2000. Influence of the level and location of contamination on the multiplication of *Salmonella enteritidis* at different storage temperatures in experimentally inoculated eggs. *Poult. Sci.* 79:559–563.
 8. Gast, R. K., and P. S. Holt. 2001. Assessing the frequency and consequences of *Salmonella enteritidis* deposition on the egg yolk membrane. *Poult. Sci.* 80:997–1002.
 9. Gast, R. K., and P. S. Holt. 2001. Multiplication in egg yolk and survival in egg albumen of *Salmonella enterica* serotype Enteritidis strains of phage type 4, 8, 13a, and 14b. *J. Food. Prot.* 64:865–868.
 10. Hammack, T. S., P. S. Sherrod, V. R. Bruce, G. A. June, F. B. Satchell, and W. H. Andrews. 1993. Growth of *Salmonella enteritidis* in Grade A eggs during prolonged storage. *Poult. Sci.* 72:373–377.
 11. Holt, P. S., H. D. Stone, R. K. Gast, and R. E. Porter, Jr. 1996. Growth of *Salmonella enteritidis* (SE) in egg contents from hens vaccinated with an SE bacterin. *Food Microbiol.* 13:417–426.
 12. Humphrey, T. J., A. Baskerville, S. Mawer, and B. Rowe. 1989. *Salmonella enteritidis* phage type 4 from the contents of intact eggs: a study involving naturally infected hens. *Epidemiol. Infect.* 103:415–423.
 13. Humphrey, T. J., A. Whitehead, A. H. L. Gawler, and A. Henley. 1991. Numbers of *Salmonella enteritidis* in the contents of intact eggs: a study involving naturally infected hens. *Epidemiol. Infect.* 103:415–423.
 14. Ibrahim, H. R., Y. Sugimoto, and T. Aoki. 2000. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochem. Biophys. Acta* 1523:196–205.
 15. Ibrahim, H. R., U. Thomas, and A. Pellegrini. 2001. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *J. Biol. Chem.* 276:43767–43774.
 16. Kido, S., M. Janado, and H. Nunoura. 1975. Macromolecular components of the vitelline membrane of hen's egg. I. Membrane structure and its deterioration with age. *J. Biochem. (Tokyo)* 78:261–268.
 17. Lu, S., P. B. Killoran, and L. W. Riley. 2003. Association of *Salmonella enterica* serovar Enteritidis YafD with resistance to chicken egg albumen. *Infect. Immun.* 71:6734–6741.
 18. Murase, T., A. Nakamura, A. Matsushima, and S. Yamai. 1996. An epidemiological study of *Salmonella enteritidis* by pulsed-field gel electrophoresis (PFGE): several PFGE patterns observed in isolates from a food poisoning outbreak. *Microbiol. Immunol.* 40:873–875.
 19. Schoeni, J. L., K. A. Glass, J. L. McDermott, and A. C. L. Wong. 1995. Growth and penetration of *Salmonella* Enteritidis, *Salmonella* Heidelberg and *Salmonella* Typhimurium in eggs. *Int. J. Food Microbiol.* 24:385–396.
 20. Shirota, K., H. Katoh, T. Murase, T. Ito, and K. Otsuki. 2001. Monitoring of layer feed and eggs for *Salmonella* in eastern Japan between 1993 and 1998. *J. Food Prot.* 64:734–737.
 21. Shott, S. 1990. Statistics for health professionals. W. B. Saunders Co., Philadelphia.